

## ***Gymnopus barbipes* and *G. dysodes*, new records for Pakistan**

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**ABSTRACT**—Morphological and phylogenetic analyses were conducted to identify *Gymnopus* species collected in Pakistan during 2013–14. Phylogenetic analysis was performed using internal transcribed spacer region (ITS) of ribosomal DNA sequences. Among the collected taxa, we identified *Gymnopus barbipes* and *G. dysodes*, represent new records for Pakistan. Their detailed descriptions and illustrations are also provided.

**KEY WORDS** —Khyber Pakhtunkhwa, *Omphalotaceae*, pine forests

## **Introduction**

*Gymnopus* (Pers.) Roussel (*Omphalotaceae*, *Agaricales*) is a large fungal genus, which consists of c. 300 species distributed worldwide (Jang & al. 2016). It is characterized by a collybioid basidioma; a convex to applanate or slightly concave pileus; free, emarginate or adnate and crowded to fairly distant lamellae; a central stipe; a white spore print; inamyloid or non-dextrinoid hyphae with clamp connections; ellipsoid to oblong, thin-walled, hyaline, inamyloid basidiospores. Cheilocystidia are often present while pleurocystidia are usually absent (Antonín & al. 2013, Jang & al. 2016, Saba & Khalid 2014). Seven species of *Gymnopus*—worldwide a relatively common

saprobe on leaf litter and dead wood—have been reported in Pakistan (Ahmad 1980, Iqbal & Khalid 1996, Shibata 1992, Sultana & al. 2011, Saba & Khalid 2014). In view of the high worldwide diversity, we would expect the diversity of Pakistani *Gymnopus* to be much greater than currently reported.

To explore the diversity of indigenous *Gymnopus* spp., we collected specimens in Pakistan, which we identified through morphological and phylogenetic analyses. Here we report *Gymnopus barbipes* and *G. dysodes* as new records for Pakistan.

## Materials & methods

### Morphological evaluation

Basidiomata were collected, photographed, characterized morphologically, dried, and deposited in the University of Swat Herbarium (SWAT) in Pakistan and Farlow Herbarium of Harvard University in USA (FH). Color designations are from Munsell (1975). Microscopical observations were made from slide preparations from dried specimens mounted in 5% potassium hydroxide (KOH) or Melzer's reagent under a Meiji Techno MX4300H biological microscope. Dimensions of anatomical features (basidiospores, basidia, cystidia, hyphae) were calculated from at least 25 measurements using an ocular micrometer and 100× oil-immersion objective with  $x$  = arithmetic means of spore lengths and widths for all spores measured. Line drawings were made with a camera lucida.

### DNA extraction, PCR amplification, DNA sequencing

Genomic DNA was extracted from a small piece of basidioma by a modified CTAB method (Lee & al. 1988) using the Qiagen DNeasy Plant Mini Kit (cat. no. 69104). The entire rDNA internal transcribed spacer region (ITS = ITS1+5.8S+ITS2) was targeted with primer pair ITS1F/ITS4 (White & al. 1990, Gardes & Bruns 1993) and then amplified using Econo Taq DNA Polymerase following the PCR cycling parameters: initial denaturation (94 °C for 1 min), 35 cycles (94 °C for 1 min, 53 °C for 1 min, 72 °C for 1 min), and final extension (72 °C for 8 min). The PCR amplicons were sent to Macrogen (Republic of Korea) for purification and bidirectional sequencing.

### Sequence alignment & phylogenetic analysis

The ITS sequence of MSM0034 comprised 700 base pairs and that of ING-21 comprised 691 base pairs. We retrieved closely related ITS sequences via a BLAST search of GenBank for subsequent phylogenetic analysis, according to Saba & Khalid (2014). Sequences used in phylogenetic construction are shown in TABLE 1.

Sequences were manually edited and assembled using BioEdit ([www.mbio.ncsu.edu/bioedit/bioedit.html](http://www.mbio.ncsu.edu/bioedit/bioedit.html)). Following Dentinger & al. (2011) for complete ITS sequences, all sequences were trimmed with the conserved motifs 5'-(...GAT) CATT- and -GACCT (CAAA...)-3' with the enclosed aligned portions analysed. Sequences of

TABLE 1: *Gymnopus* and *Omphalotus* ITS sequences analyzed.  
New sequences in **bold**.

| TAXON                      | COUNTRY            | GENBANK #       | COLLECTION/VOUCHER # |
|----------------------------|--------------------|-----------------|----------------------|
| <i>G. barbipes</i>         | <b>Pakistan</b>    | <b>MK450334</b> | <b>MSM 0034</b>      |
|                            | USA                | KJ416265        | TENN <b>69173</b>    |
|                            | USA                | KJ416266        | TENN <b>69173</b>    |
|                            | USA                | KJ416269        | TENN <b>67858-T</b>  |
| <i>G. biformis</i>         | Costa Rica         | DQ450064        | TFB7843              |
|                            | Costa Rica         | DQ450063        | TFB7820              |
| <i>G. confluens</i>        | Russia             | AY256697        | TENN58242            |
|                            | —                  | HM240527        | UBC F19677           |
| <i>G. cylindricus</i>      | Costa Rica         | AY256696        | TENN58024            |
|                            | Costa Rica         | DQ450057        | TFB10091             |
| <i>G. dryophilus</i>       | Italy              | JX536157        | BRNM707149           |
| <i>G. dysodes</i>          | <b>Pakistan</b>    | <b>MT114698</b> | <b>SWAT 001355</b>   |
|                            | USA                | AF505778        | TENN 59141           |
|                            | USA                | KY026666        | TENN F-61125         |
|                            | Czech Republic     | JX536158        | BRNM712600           |
| <i>G. erythropus</i>       | Slovakia           | DQ449996        | SAV XI2002           |
|                            | USA                | DQ449998        | WTU JFA12910         |
| <i>G. fibrosipes</i>       | Costa Rica         | AF505763        | TENN56660            |
|                            | —                  | AY842953        | PR23TN               |
| <i>G. fusipes</i>          | France             | AY256710        | TENN59217            |
|                            | Austria            | AF505777        | TENN59300            |
| <i>G. gibbosus</i>         | —                  | AY263437        | AWW66                |
|                            | —                  | AY263438        | AWW95                |
| <i>G. impudicus</i>        | Russia             | KJ416263        | TENN 60094           |
|                            | Russia             | KJ416264        | Culture              |
| <i>G. luxurians</i>        | Pakistan           | KF803760        | MSM 001              |
|                            | Pakistan           | KF803761        | MSM 002              |
|                            | USA                | AY256709        | TENN57910            |
|                            | Switzerland        | DQ450022        | TENN50619            |
| <i>G. menehune</i>         | Pakistan           | KF803762        | MSM 003              |
|                            | —                  | JN182864        | —                    |
|                            | —                  | AY263426        | DED5866              |
| <i>G. mesoamericanus</i>   | Costa Rica         | DQ450036        | TENN58106            |
|                            | Costa Rica         | AF505768        | NYBG REH7379         |
| <i>G. polygrammus</i>      | Puerto Rico        | DQ450028        | TENN56589            |
|                            | —                  | AY842954        | PR 2542TN            |
| <i>G. readiae</i>          | New Zealand        | DQ450034        | TENN53687            |
|                            | New Zealand        | HQ533036        | PDD95844             |
| <i>G. subcylathiformis</i> | Puerto Rico        | DQ450041        | TENN58130            |
|                            | Dominican Republic | DQ450042        | TENN59550            |
| <i>G. subpruinosis</i>     | USA                | DQ450026        | TENN56242            |
|                            | USA                | DQ450027        | TENN59477            |
| <i>O. illudens</i>         | USA                | AY313271        | TENN54507            |
| <i>O. olearius</i>         | France             | AY313277        | Culture 9061b        |
| <i>O. olivascens</i>       | USA                | AY313281        | TENN55337            |

*Omphalotus* spp. were used as outgroup based on results reported by Moncalvo & al. (2002) and Mata & al. (2004).

We aligned our new sequences with GenBank sequences from related taxa using ClustalX (Thompson & al. 1997) and manually edited where necessary using BioEdit (Hall 1999). A maximum likelihood analysis was performed via CIPRES Science Gateway (Miller & al. 2010) employing RAxML-HPC v.8 and Rapid bootstrap analysis/search for best scoring, with the topology assessed by 1000 bootstrap replicates.

## Taxonomy

*Gymnopus barbipes* R.H. Petersen & K.W. Hughes,

N. Amer. Fung. 9(3): 2 (2014).

FIG. 1

Pileus 25 mm diam., broadly convex, expanding with age to plane, flat, thin; margin straight; surface matte, smooth; central disc brownish orange (5YR5/8) to strong brown (5YR4/6) or light brown (5YR8/8). Lamellae adnexed or free, subdistant, light orange (2.5YR8/8), edges even, concolorous. Lamellulae 1–2 tiered. Stipe 40 × 5 mm, central, equal, cylindrical, hollow, sheathed in a thin, off-white, felty covering, brownish orange (5YR5/8). Odor & taste not distinctive.

Basidiospores 5–7 × 2.3–4 µm [ $\bar{x}$  = 5.6 × 3.0 µm,  $Q$  = 1.2–1.6], ellipsoid in profile view, slightly flattened adaxially, rarely ovoid, smooth, thin-walled, aguttulate, hyaline in KOH, inamyloid. Basidia 23–28 × 5–6 µm, clavate, 4-spored (rarely 2-spored) basidia, thin-walled, hyaline in KOH; sterigmata 3–3.7 µm. Basidioles subclavate, abundant. Pleurocystidia absent. Cheilocystidia abundant, sometimes constricted at neck. Pileipellis a cutis, repent hyphae cylindrical, 5–14 µm, thin-walled, hyaline in KOH. Stipe hyphae cylindrical, 5–9 µm, non-encrusted, hyaline in KOH. Caulocystidia cylindrical, knobby, abundant, hyaline, thin-walled, 9–14 µm in diam. Clamp connections present in all tissues.

MATERIAL STUDIED: PAKISTAN, KHYBER PAKHTUNKHWA, Shangla, Yakh Tangay, under *Pinus wallichiana* A.B. Jacks., 2 September 2013, Malka Saba & Abdul Nasir Khalid, MSM#0034 (FH00304574; GenBank MK450334).

COMMENTS—Our Pakistani specimen agrees morphologically with the original protologue of *G. barbipes* except for its broader pileus (cited as 5–15 mm diam. in Petersen & Hughes 2014), robust stipe, and presence of ovoid basidiospores in addition to ellipsoid basidiospores. *Gymnopus barbipes* was described from southeastern North America and characterized by its occurrence on leaf litter and absence of well-developed pleurocystidia and cheilocystidia (Petersen & Hughes 2014). Our Pakistani collection



FIG. 1. *Gymnopus barbipes* (MSM 0034). A, B. Basidiomata; C. Basidia; D. Cheilocystidia; E. Basidiospores; F. Caulocystidia; G. Pileipellis. Scale bars: A, B = 10 mm; C, D = 10 µm; E = 5 µm; F, G = 25 µm.

represents the first occurrence of *G. barbipes* reported since the Tennessee collection was described.

*Gymnopus dysodes* (Halling) Halling, Mycotaxon 63: 364 (1997).

FIG. 2

≡ *Collybia dysodes* Halling, Mycol. Mem. 8: 79 (1983).

Pileus 15–30 mm across, conic to campanulate at first, later plano-convex to plane, color light pinkish brown (5YR 6/4) to pale reddish brown (5YR 4/4) with a darker (7.5YR 1/2) disc, surface dry, dull, disc smooth, otherwise sulcate, pileus margin crenate or eroded in some specimens, context thin, 1–2 mm at the disc, ≤1mm at the margins, flesh pinkish brown (7.5YR 9/4), unchanging when cut. Lamellae adnexed, seceding, narrow (1.5–2.5 mm) wide, distant, initially creamy pinkish (5Y 9/4), becoming light brown (7.5YR 7/4) and finally dark brown (5Y 3/4). Lamellulae present, rare, short, mostly in single tier. Stipe 10–20 × 4–7 mm, cylindrical in young specimens, compressed in mature specimens, equal to thickening downward, surface reddish brown (7.5 YR 9/4), smooth or slightly scaly with hirsute base, interior hollow at the center, pith at apex and in base whitish fibrillose, concolorous with stipe surface, unchanging when cut. Odor of garlic. Taste not noted.

Basidiospores  $7.3\text{--}8.6 \times 3.4\text{--}3.9 \mu\text{m}$ , [ $x = 7.9 \times 3.7 \mu\text{m}$ ,  $Q = 1.8\text{--}2.4$ ], narrowly ellipsoid to oblong, smooth, thin-walled, hyaline in both KOH and Congo Red, inamyloid. Basidia  $22\text{--}25 \times 4\text{--}6 \mu\text{m}$ , clavate, with four sterigmata, thin-walled, hyaline in KOH. Basidioles subclavate to cylindrical, numerous. Cheilocystidia  $25\text{--}35 \times 5\text{--}7 \mu\text{m}$ , variously shaped, cylindrical, narrowly utriform or fusoid, flexuous, bottle shaped, horned/rostrate, thin-walled, clamped at bases, hyaline in KOH. Pleurocystidia absent. Pileipellis a cutis, individual hyphae  $5\text{--}10 \mu\text{m}$  diam., terminals mostly acute, some cylindrical, others with irregular terminals, thin-walled.

MATERIAL STUDIED: PAKISTAN, KHYBER PAKHTUNKHWA, District Swat, village Ingaro Dherai, in clusters on decaying woodchips of deciduous trees, 23 July 2014, Junaid Khan, ING-21 (SWAT 001355; GenBank MT114698).

COMMENTS—Our Pakistani specimen agrees morphologically with the original protologue of *Collybia dysodes* by Halling (1983). *Gymnopus dysodes* falls within *G. sect. Impudici*, characterized by an unpleasant smell resembling rotten cabbage, onion, garlic, etc.

*Gymnopus dysodes*, a North American species, is characterized mainly by its lignicolous habit, dark reddish brown to cinnamon brownish basidiomata, cinnamon brown lamellae, dark to light reddish brown stipe, a pungent

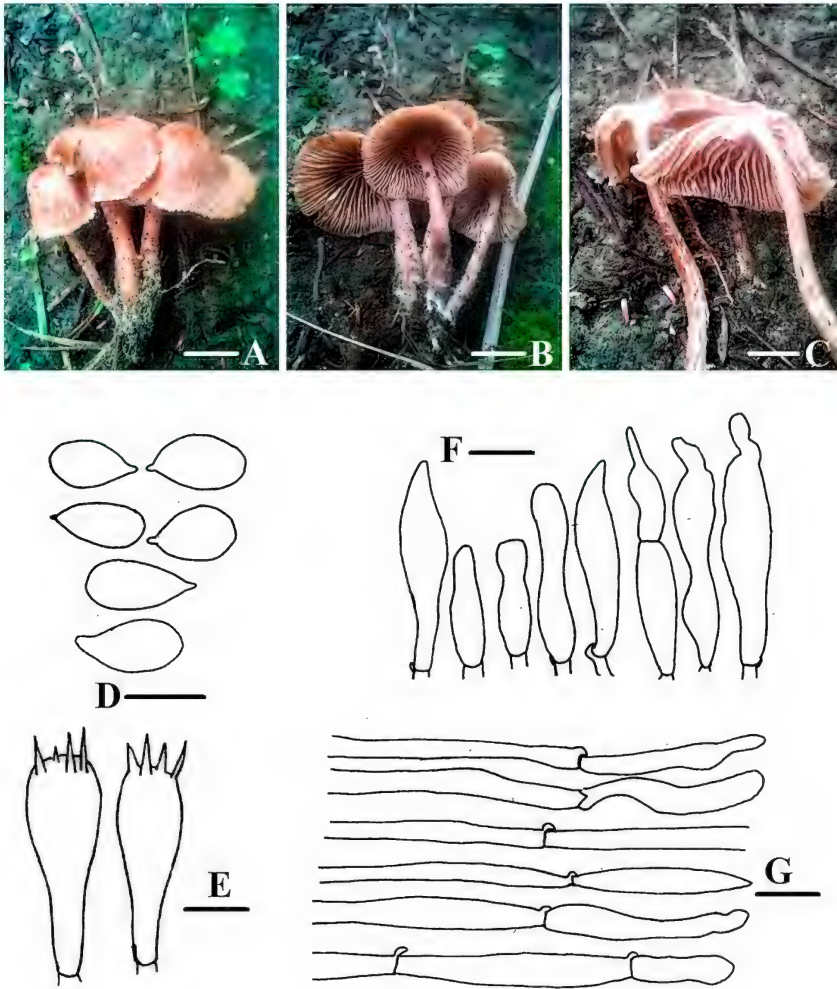


FIG. 2. *Gymnopus dysodes* (ING-21). A–C. Basidiomata; D. Basidiospores; E. Basidiole and basidium; F. Cheilocystidia; G. Pileipellis hyphae (with clamped septa). Scale bars: A–C = 5 mm; D, E = 5  $\mu$ m; F = 10  $\mu$ m; G = 15  $\mu$ m.

garlic-like odour, and cylindrical to flexuous cheilocystidia (Halling 1983). Our collection was also collected on decaying wood chips of deciduous trees; both morphology and sequence analysis support its identification as



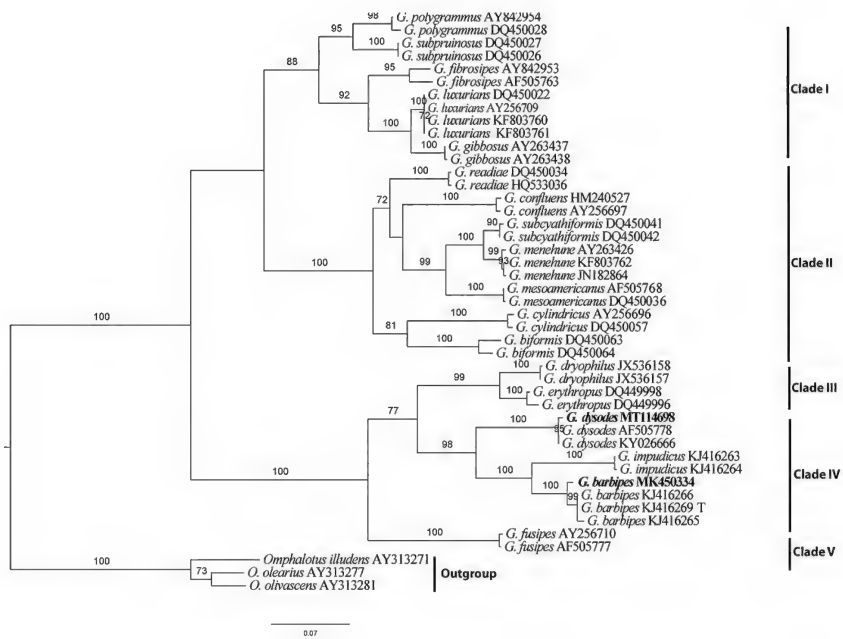


FIG. 3. Molecular phylogenetic analysis of *Gymnopus* sequences by the Maximum Likelihood method. The bootstrap consensus tree inferred from 1000 replicates implies the evolutionary history. The percentage of trees in which the taxa cluster together is shown next to the branches. New sequences reported in this study are in **bold**; T = holotype.

*G. dysodes*. There are no previous reports of *G. dysodes* from the country, which makes this species an addition to the fungi of Pakistan.

*Gymnopus impudicus* (Fr.) Antonín & al., a similar species, can be separated by its darker basidiomata (dark brown pileus and almost black stipe base), fetid (not garlic-like) odour, smaller basidiospores ( $5.5\text{--}7.5 \times 3.5\text{--}4\text{ }\mu\text{m}$ ), and moniliform to coralloid cheilocystidia ( $20\text{--}40 \times 3\text{--}9\text{ }\mu\text{m}$ ; Noordeloos 1995).

### Phylogeny

A total of 45 nrITS sequences were used in the molecular phylogenetic analysis, 42 representing 18 *Gymnopus* taxa and three representing three *Omphalotus* in-group taxa (TABLE 1). The final dataset had 935 sites of which 475 were conserved, 454 were variable, and 423 were parsimony informative.



Maximum likelihood analysis clustered the included nucleotide sequences of *Gymnopus* into five well supported clades. Species within the Clades I and II are members of *Gymnopus* subsect. *Vestipedes*. Clade III correlates to *G.* subsect. *Levipedes*, clade IV to *G.* subsect. *Impudici*, and clade V to *G.* sect. *Gymnopus*. Both Pakistani species fall within clade IV (*G.* subsect. *Impudici*). *Gymnopus dysodes* and *G. barbipes* clustered with sequences from USA with strong bootstrap support (100%).

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